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Will temperature and salinity changes exacerbate the effects of seawater acidification on the marine microalga *Phaeodactylum tricornutum*?

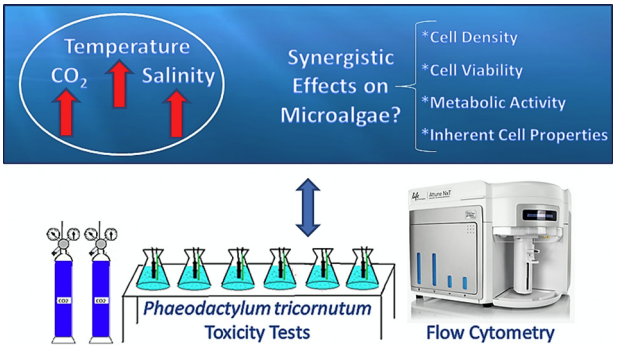
Esther Bautista-Chamizo [a,*], Marta Sendra [b], Ángeles Cid [c], Marta Seoane [c], Manoela Romano de Orte [d,e], Inmaculada Riba [a]

[a] Departamento de Química-Física, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Spain
[b] Departamento de Ecología y Gestión Costera, Instituto de Ciencias Marinas de Andalucía (CSIC), Spain
[c] Departamento de Biología, Facultad de Ciencias, Universidade da Coruña, Spain
[d] Departamento de Ciências do Mar, Campus Baixada Santista, Universidade Federal de São Paulo, Brazil
[e] Department of Global Ecology, Carnegie Institution for Science, Stanford, USA

HIGHLIGHTS

- Combined effects of pH, temperature and salinity were studied on a marine diatom.
- A novel CO_2 injection system was used for performing microalgae toxicity test.
- Synergistic effects were found on cell viability, cell size and autofluorescence.
- Results are useful to address the potential impact of climate change.

GRAPHICAL ABSTRACT



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ABSTRACT

To evaluate the effects related to the combination of potential future changes in pH, temperature and salinity on microalgae, a laboratory experiment was performed using the marine diatom *Phaeodactylum tricornutum*. Populations of this species were exposed during 48 h to a three-factor experimental design ($3 \times 2 \times 2$) with two artificial pH values (6, 7.4), two levels of temperature (23 °C, 28 °C), two levels of salinity (34 psu, 40 psu) and a control (pH 8, Temp 23 °C, Sal 34 psu). The effects on growth, cell viability, metabolic activity, and inherent cell properties (size, complexity and autofluorescence) of *P. tricornutum* were studied using flow cytometry. The results showed adverse effects on cultures exposed to pH 6 and high temperature and salinity, being the inherent cell properties the most sensitive response. Also, linked effects of these parameters resulted on cell viability and cell size decrease and an increase of cell autofluorescence. The conclusions obtained from this work are useful to address the potential effects of climate change (in terms of changes on pH, salinity and temperature) in microalgae.

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1. Introduction

Climate change will influence oceans and coasts in different ways (Philippart, 2007). The rising of temperature is one of the most evident

* Corresponding author.
E-mail address: esther.bautista@uca.es. (E. Bautista-Chamizo).

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consequences of this process, and it is the reason why glaciers and the Greenland ice cover are melting (Teng et al., 2006). The effects will be of a great magnitude: sea level rise, floodings (Frei et al., 2006), heat waves, hurricanes, droughts and other extreme events (IPCC, 2007). These global environmental changes are happening at an exceptional speed with no precedents in the geological past (IPCC, 2014). Moreover, climate change will have other negative impacts apart from extreme weather events; it will also cause changes in terrestrial and marine ecosystems (Root et al., 2003; Svenning et al., 2009).

Oceans, in their role of carbon dioxide (CO_2) sinks, are acting as a buffer of climate change (Egilsdottir et al., 2009). However, as a result of this CO_2 absorption, the ocean chemistry is being altered, with a decrease of the seawater pH (Raven, 2005). Ocean pH has fallen about 0.1 units since pre-industrial times (Orr et al., 2005) and it is predicted to decrease about 0.3–0.5 units by the year 2100 and 0.5–0.7 units by the year 2300 (Caldeira and Wickett, 2003). In order to mitigate climate change by reducing the CO_2 already available in the atmosphere, the Carbon Capture and Storage technology is getting importance as a short-term solution (IPCC, 2014). By injecting CO_2 in the sub-seabed storage sites, this technology has the capacity to capture 40 million tons of CO_2 worldwide (Global CCS Institute, 2016). Yet, this technology has some associated risks, such as a potential CO_2 leakage, which could lead to an extreme acidification of the surrounding area of the escape, reaching pH values of 6.0 or even lower (Santana-Casiano et al., 2012). Seawater acidification has a wide and complex variety of responses within marine organisms and adverse effects are not necessarily observed, since sensitivities to acidification are species, population and even life-stage specific (Ries et al., 2009; Wang et al., 2015).

Global average temperature has increased by 0.7 °C during this last century (Hansen et al., 2006) and according to IPCC (2007) it is predicted to keep rising between 1.8 °C and 4 °C by the end of this century. Salinity is expected to change as a result of this global warming phenomenon, being reduced or increased depending on the region latitude.

This environmental problem has been addressed by many authors in studies dealing with the effects of seawater acidification on marine higher organisms (Basallote et al., 2012; Rodríguez-Romero et al., 2016), microalgae (Iglesias-Rodriguez et al., 2008; Müller et al., 2010; Johnson et al., 2013; Li et al., 2017) and bacteria (Borrero-Santiago et al., 2016a, 2016b; Díaz-García et al., 2016). However, current studies rarely take into account the potential synergistic effects of environmental factors under climate change scenarios. Only a few research studies are focused on the effects of CO_2 enrichment when combined with changes on temperature or salinity (Matozzo et al., 2012; Ko et al., 2014; Pereira et al., 2016), but there is still a lack of research about the potential effects of these three combined environmental factors on microalgae.

Diatoms are ubiquitous marine photosynthetic eukaryotes responsible for approximately 20% of global primary productivity, playing a central role in the biogeochemical cycling of important nutrients (Rosenwasser et al., 2014). *Phaedactylum tricornutum* (Bacillariophyceae) (Bohlin, 1897), which has been found in great abundance in coastal and oceanic waters, was selected for the study as it is considered a model organism in ecotoxicological studies (OECD, 1984; USEPA, 1985; ISO, 1987) and also because of its importance in aquaculture as nutrient for higher organisms.

This work hypothesizes that the combination of pH, temperature and salinity changes could cause unwanted effects in marine phytoplankton. Therefore, the objective of this work is to provide an integrated knowledge of the microalgae response to future and potential global warming, seawater acidification and increased salinity. Such understanding will be of great importance in order to manage and fight climate change effects on marine ecosystems. For this purpose, three pH values (6, 7.4, and a non-acidified control pH 8), two temperature values (23 °C, 28 °C) and two salinity values (34 psu, 40 psu) were selected to investigate the effects on key physiological parameters of the microalgae *P. tricornutum*.

2. Material and methods

2.1. Sampling and algae culture conditions

Seawater was collected from Río San Pedro, in the Bay of Cádiz (Atlantic coast of South-West Spain) as this location has been proved to be a valid reference station in many previous ecotoxicology works (Riba et al., 2004; Basallote et al., 2014; Borrero-Santiago et al., 2017). The natural properties of this seawater were: pH of 8, temperature 23 °C and salinity of 34 psu (standard deviation ± 0.1). *P. tricornutum* was obtained from Servicios Centrales de Investigación en Cultivos Marinos from the University of Cádiz. Stock cultures were cultivated using this seawater (pH 8, Temp 23 °C, Sal 34 psu) under a 24 h light regime.

2.2. CO_2 injection system

In order to reproduce an event of seawater acidification or a potential CO_2 leakage, a CO_2 injection was used. This system (patent no: ES2618843) described by Borrero-Santiago et al. (2016ab), is inside of a non-pressurized chamber, and it has been designed to guarantee the sterile conditions during the development of the toxicity test. It also permits the control of other parameters such as temperature and light. Briefly, the semi-automatic system consists on the injection of CO_2 through silicone hoses regulated by an AT control software (Aqua Medic 8.0), which acts according to the data received from the pH meters. The precision of the pH values has a minimal variety of ±0.1. The experimental set-up was developed according to Bautista-Chamizo et al. (2016).

2.3. Toxicity tests

Toxicity tests were performed in triplicate and using sterilized Erlenmeyer flasks, previously washed with HNO_3 (10%) and autoclaved. Continuous illumination was provided by six fluorescent lights (36 W). Each flask was filled with 150 mL of filtered (Millipore 0.22 μm) seawater to avoid bacteria and other microalgae, enriched with f/2 medium (Sunda and Guillard, 1976) and silicate. Flasks were shaken at 4-h intervals to avoid cell precipitation and flasks position was changed with respect to the light source at 8-h intervals to ensure that all of them received the same level of illumination.

Exponentially-growing (72 h) populations of microalgae were exposed to a three factor experimental design ($3 \times 2 \times 2$) with two artificial pH values (6,7.4), adjusted by CO_2 injection, and a control (non-acidified natural seawater, pH 8), two levels of temperature (23 °C, 28 °C) and two levels of salinity (34 psu, 40 psu) (standard deviation 0.1). The selected pH values are related to two different scenarios; a predicted future value (pH 7.4) and the scenario of a potential CO_2 leakage (pH 6). The experimental stressors represent the average surface water temperature and salinity of the study area (23 °C, 34 psu) and the potential future scenarios of temperature (28 °C) and salinity (40 psu). All the possible combinations were tested, with a result of twelve treatments, which are detailed as follows: single stressor treatments (testing changes on pH, temperature (Temp) and salinity (Sal)), dual combination of stressors treatments (testing changes on: pH × Temp, pH × Sal, Sal×Temp) and a triple combination of stressors treatments (pH × Temp×Sal) being the combination: pH 8, Temp 23 °C and Sal 34 psu our control.

The initial cell density was established at 3×10^4 cells mL^{-1}. The experiments were run for 48 h. After this short time period an entire cell cycle is able to be completed (Seoane et al., 2017) and microalgae are able to experience metabolic adjustment in response to stressors (Esperanza et al., 2015). All the parameters were measured at the end of the experiment with a flow cytometer. Toxicity tests were developed according to international standards (OECD, 1984).

Desirable pH values were maintained by bubbling CO_2 into the water. High salinity was achieved by evaporation, and adjusted by

means of distilled water. Water temperature was maintained by a heater with thermostat. Both pH and temperature were monitored in continuous, and salinity was checked daily using a hand refractometer (50302020 Zuzi) and a portable conductivity meter (Sension+ EC5 Portable Conductivity Kit, Hach).

2.4. Seawater carbonate chemistry

Alkalinity measurements were performed at the end of the experiment with an automatic alkalinity titrator (Metler Toledo, T50). Water samples (50 mL) were taken and filtered through a 45 μm syringe filter. Carbonate system speciation was calculated using the measured values of pH, temperature, salinity and total alkalinity, with the CO2SYS software (Pierrot et al., 2006) with dissociation constant from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and potassium bisulfate ($KHSO_4$) according to Dickson (1990).

2.5. Flow cytometry analyses

Flow cytometry analyses (FCM) were performed in an Attune® NxT Acoustic Focusing Cytometer (Thermo Fisher) equipped with a 488 nm excitation argon laser, detectors of forward (FS) and side (SS), light scatter and four fluorescence channels corresponding to four wavelength intervals: FL1 (505–550 nm), FL2 (550–600 nm), FL3 (600–645) and FL4 (>645 nm). The cytometer is controlled by the Attune® NxT Software. For each parameter investigated, at least 50,000 gated cells were collected per sample. To exclude non-algal particles, microalgae autofluorescence was used as a reference (Prado et al., 2012a). Except for the inherent cell properties, the analyzed parameters (cell density, cell viability and metabolic activity) were determined using different functional fluorochromes. Aliquots of the reagents were prepared prior to the experiments, dissolved in DMSO and stored at −20 °C.

2.5.1. Cell density

Samples were taken after 48 h and immediately analyzed by FCM. Each replicate was sampled three times and 100 μL of sample were measured by the counting cells function of the flow cytometer.

2.5.2. Cell viability

Variations of cell viability through different pH treatments were studied through the staining of cells with propidium iodide (PI) from Sigma Aldrich, at a final concentration of 4 μM for 15 min at room temperature and in darkness. This fluorochrome is able to penetrate just on those cells with damaged membrane, being stuck to nucleic acid structures. Thus, it is possible to distinguish non fluorescent viable cells from fluorescent nonviable cells, using the FL3 channel. Thermal shock dead cells by microwave heating were used as a positive control (Prado et al., 2009).

2.5.3. Metabolic activity

Metabolic activity was calculated by cell staining with fluorescein diacetate (FDA). Fluorescein is accumulated by active cells and it has been previously demonstrated as a rapid and effective technique to assess microalgae metabolic activity (Jochem, 1999).

This protocol was performed following the method described by Prado et al. (2009). Briefly, samples were stained with a final FDA concentration of 0.05 μg mL^{-1} (15 min, room temperature and darkness). Then, fluorescence was detected in the FL1 channel. Results are expressed as a percentage of metabolically active cells. Thermal shock dead cells by microwave heating were used as a negative control.

2.5.4. Inherent cell properties: Cell size, complexity and autofluorescence

All these parameters were measured right after taking the sample, without the use of fluorochromes. Mean values were obtained in logarithmic scale, and represented in arbitrary units (a.u.).The information related to cell size was given by the forward light scatter (FS) since the intensity of this detector was correlated with the cell size or volume (Mullaney et al., 1969). On the other hand, the side light scatter (SS) was correlated with the intracellular complexity (Shapiro, 1995). Autofluorescence, related to chlorophyll *a* fluorescence emission, was measured with the FL3 channel to study the potential changes on pigments.

2.5.5. Statistical analysis

Confidence intervals (95%) were firstly calculated in order to evaluate the significant differences among all the treatments compared to the control (pH 8, Temp 23 °C, S 34 psu). This procedure is statistically robust and easy to interpret. The effects of the three stressors (pH, temperature and salinity) and their interactions were also analyzed using a three-way ANOVA. Prior to this analysis, normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were checked. The software SPSS 15.0 was used to perform statistical analyses.

3. Results

3.1. Seawater carbonate chemistry

Carbon parameters were calculated based on initial values of pH (6, 7.4 and 8), temperature (23 °C, 28 °C), salinity (34 psu, 40 psu) and total alkalinity (Table 1). During the development of the experiments, the values of pH, temperature and salinity did not deviate substantively from the target levels in any of the treatments. Total inorganic carbon (TIC) and pCO_2 were higher as pH was reduced. All decreased pH treatments and their combinations with high temperature and salinity treatments led to calcite and aragonite undersaturation ($\Omega_{Cal} < 1$; $\Omega_{Arag} < 1$), being the decrease more abrupt on pH 6 treatments, where the calcite and aragonite saturation index values were around zero.

3.2. Cell density

After 48 h of exposure, *P. tricornutum* cultures from the low pH treatments exhibited a lower cell density than the control (Fig. 1). Significant differences ($p < 0.05$) were found on all the combination treatments except for pH 7.4 (Temp 23 °C, Sal 40 psu). The maximum decrease was detected on pH 6 (Temp 28 °C) where the number of cells was reduced around three times under salinity 34 psu and 40 psu. The three-way ANOVA results (Table 2) showed that the combinations of pH × Temp and pH × Sal significantly influenced on *P. tricornutum* cell density. In this case, the significant difference for the triple combination pH × Temp × Sal was not applicable (N.A.) since the maximum significance was found on two-way ANOVA (Table 2).

3.3. Cell viability

Cell viability in *P. tricornutum* was significantly ($p < 0.05$) reduced on cultures under pH 6 treatments (Temp 23–28 °C and Sal 34–40 psu) (Fig. 1). The three-way ANOVA results showed a significant influence of pH × Temp × Sal on cell viability ($F = 8.479$; $p < 0.05$) (Table 2).

3.4. Metabolic activity

After 48 h of exposure, the esterase activity analysis revealed that the cell metabolic activity seems to be exacerbated by high temperature and salinity. Results showed significant effects ($p < 0.05$) on pH 8 (Temp 28 °C, Sal 40), pH 7.4 (Temp 28 °C, Sal 40 psu) and pH 6 treatment cultures (Temp 28 °C, Sal 34–40 psu) (Fig. 1). Significant differences (three-way ANOVA) were found on the dual combination pH × Sal ($F = 3.892$, $p < 0.05$) (Table 2).

Table 1
Carbonate system speciation in seawater for each pH (pH 6, pH 7.4, control pH 8), temperature (23 °C and 28 °C) and salinity (34 psu and 40 psu) treatment and their interactions. Abbreviations: total alkalinity (TA), total inorganic carbon (TIC), bicarbonate ion concentration (HCO_3^-), carbonate ion concentration (CO_3^{2-}), carbon dioxide concentration (CO_2), partial pressure of carbon dioxide (pCO_2), calcite saturation state (Ω_{Cal}) and aragonite saturation state (Ω_{Arag}). Abbreviations for the treatments: T23-S34 (Temp 23 °C, Sal 34 psu), T28-S34 (Temp 28 °C, Sal 40 psu), T23-S40 (Temp 23 °C, Sal 40 psu) and T28-S40 (Temp 28 °C, Sal 40 psu).

Treatment	TA (μmol/kg SW)	TIC (μmol/kg SW)	HCO_3^- (μmol/kg SW)	CO_3^{2-} (μmol/kg SW)	CO_2 (μmol/kg SW)	pCO_2 (μatm)	Ω_{Cal}	Ω_{Arag}
pH 6								
T23-S34	2774.3	5551.01	2770.5	2.0	2778.5	92,769	0.05	0
T28-S34	2468	4770.3	2464	2.1	2304.2	87,186.2	0.05	0.03
T23-S40	2794.7	5430.15	2790.1	2.34	2637.7	90,905.2	0.05	0
T28-S40	2575.7	4848.9	2570.7	2.5	2275.7	88,763.9	0.06	0.04
pH 7.4								
T23-S34	2682.4	2676.3	2587.8	45.6	101.95	3536	1.29	0.79
T28-S34	2497.4	2515.44	2376.6	50.5	88.5	3347.7	1.23	0.82
T23-S40	2915.2	2938.5	2775.5	58.5	104.4	3600	1.35	0.9
T28-S40	2558	2558.5	2414.3	59.1	85.1	3318.8	1.37	0.91
pH 8								
T23-S34	2798.9	2586.4	2389.5	173.0	23.9	800.1	4.19	2.74
T28-S34	2621.5	2386.6	2182.0	184.2	20.4	772.1	4.50	2.99
T23-S40	3202.1	2923.1	2673.4	224.5	25.3	871	5.17	3.40

3.5. Inherent cell properties

Inherent cell properties results are shown in Fig. 2. The most sensitive parameter was autofluorescence, which was significantly increased in all cultures exposed to the different pH treatments under temperature 28 °C (Sal 34–40 psu) and pH 6–7.4 (Temp 23 °C, Sal 34 psu). Cell size was significantly increased at pH 7.4 and 6 (Temp 23 °C, Sal 34 psu) with respect to the control. In both parameters, acidification effects seem to be mitigated under high salinity (Temp 23 °C). An increase of intracellular complexity

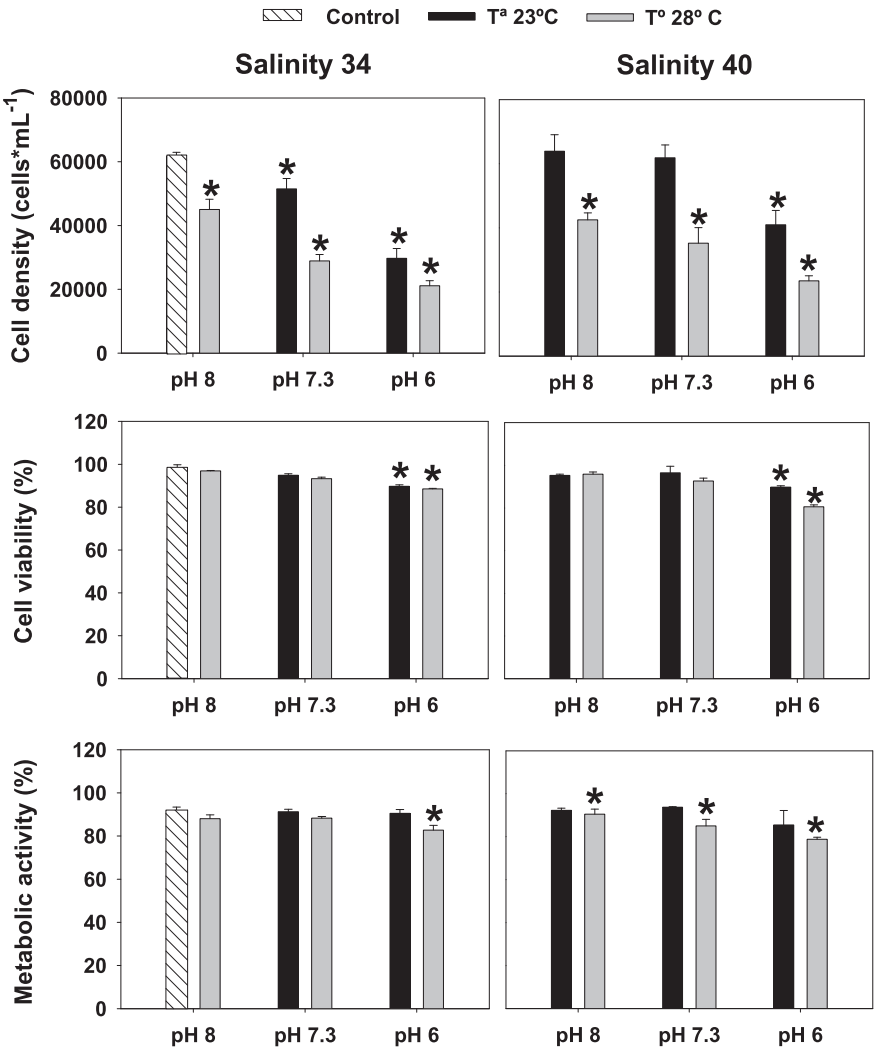

Fig. 1. Data represent the mean values for *P. tricornutum* cell density, cell viability and metabolic activity under each treatment combination (standard deviation $n = 3$). Panel A represent salinity 34 psu and panel B represents salinity 40 psu. Significant differences ($^*p < 0.05$) have been calculated comparing with the control (pH 8, Temp 23 °C, Sal 34 psu).

Table 2
Results of three-way Anova showing the F ratios and *p* values for the interactive effects of pH, temperature (Temp) and salinity (Sal) on growth, cell viability, metabolic activity (Met. Act.), cell size, cell complexity (Complex.), and autofluorescence (Autofl.). Significant effects ($p < 0.05$) are indicated in bold.

Parameter	pH	Temp	Sal	pH × Temp	pH × Sal	Temp×Sal	pH × Temp×Sal
Growth	**$F_2 = 105.32$**	**$F_1 = 204.39$**	**$F_1 = 19.002$**	**$F_2 = 8.918$**	**$F_2 = 4.008$**	$F_1 = 4.053$	N.A
	$p = 0.00$	**$p = 0.00$**	**$p = 0.001$**	**$p = 0.001$**	**$p = 0.001$**	$p = 0.055$	
Viability	**$F_2 = 179.02$**	**$F_1 = 53.406$**	**$F_1 = 24.701$**	**$F_2 = 16.328$**	**$F_2 = 13.67$**	**$F_1 = 8.563$**	**$F_2 = 8.479$**
	$p = 0.00$	**$p = 0.00$**	**$p = 0.00$**	**$p = 0.00$**	**$p = 0.00$**	**$p = 0.007$**	**$p = 0.002$**
Met. Act.	**$F_2 = 19.131$**	**$F_{1=}35.511$**	$F_1 = 1.636$	$F_2 = 2.193$	**$F_2 = 3.892$**	$F_1 = 0.222$	N.A
	$p = 0.00$	**$p = 0.00$**	$p = 0.212$	$p = 0.132$	**$p = 0.033$**	$p = 0.642$	
Cell size	**$F_2 = 25.778$**	**$F_1 = 174.35$**	**$F_1 = 13.545$**	**$F_2 = 35.247$**	**$F_2 = 20.200$**	**$F_1 = 8.495$**	**$F_2 = 21.577$**
	$p = 0.00$	**$p = 0.00$**	**$p = 0.001$**	**$p = 0.00$**	**$p = 0.00$**	**$p = 0.008$**	**$p = 0.00$**
Complex.	**$F_2 = 4.652$**	**$F_1 = 308.91$**	$F_1 = 3.175$	$F_2 = 0.944$	$F_2 = 0.309$	**$F_1 = 6.701$**	N.A
	$p = 0.019$	**$p = 0.00$**	$p = 0.086$	$p = 0.402$	$p = 0.737$	**$p = 0.016$**	
Autofl.	**$F_2 = 58.44$**	**$F_1 = 1814.9$**	$F_1 = 1.699$	**$F_2 = 17.908$**	**$F_2 = 9.958$**	**$F_1 = 9.799$**	**$F_2 = 7.294$**
	$p = 0.00$	**$p = 0.00$**	$p = 0.205$	**$p = 0.00$**	**$p = 0.001$**	**$p = 0.005$**	**$p = 0.003$**

was also observed in cultures exposed to the different pH treatments, at temperature 28 °C. Cell size ($F = 21.577$) and autofluorescence ($F = 7.294$) were significantly ($p < 0.05$) affected by the triple combination of pH, temperature and salinity, while cell complexity was significantly influenced just by the combination of salinity and temperature ($F = 6.701$, $p < 0.05$).

4. Discussion

In the present work, the combined effects of potential future values of pH, temperature, and salinity on *P. tricornutum* were evaluated to understand the potential synergetic effects of these three variables, which are deeply related to each other as a consequence of climate change.

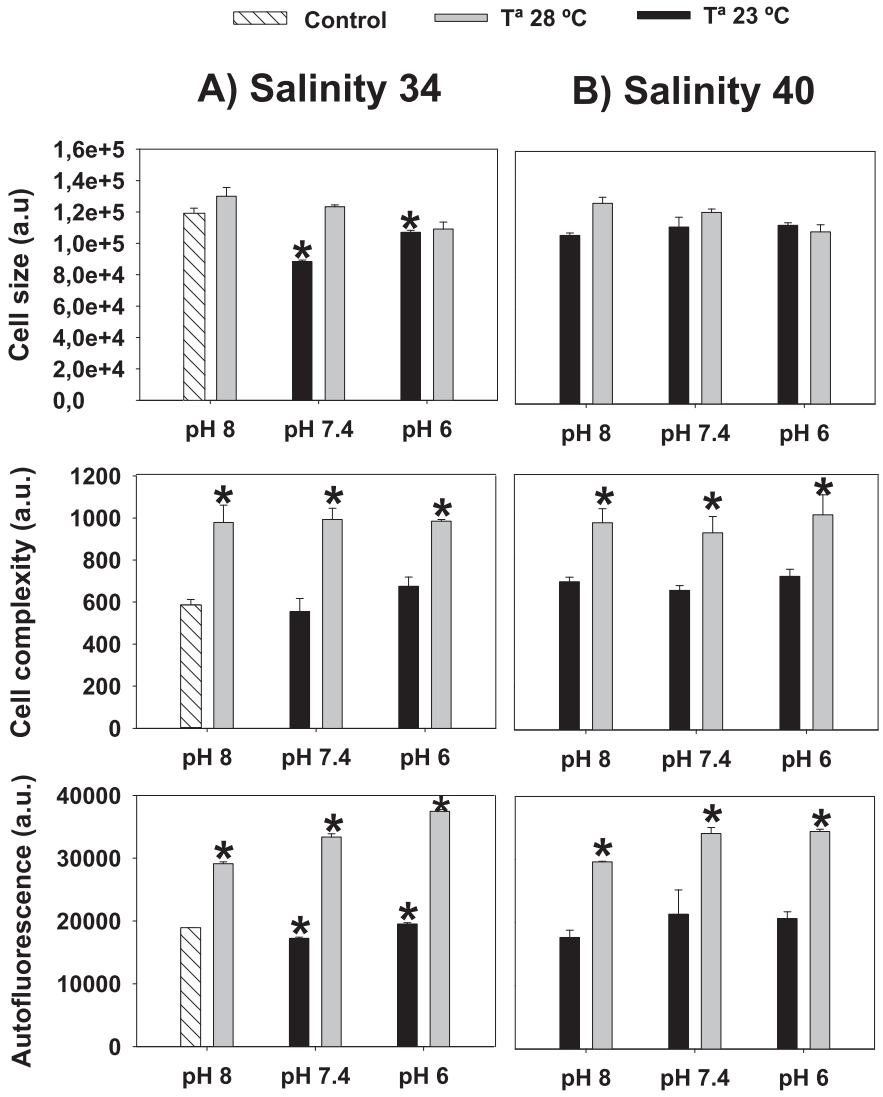

Fig. 2. Data represent the mean values for *P. tricornutum* cell size, cell complexity and autofluorescence under each treatment combination (standard deviations $n = 3$). Panel A represent salinity 34 psu and panel B represents salinity 40 psu. Significant differences ($^*p < 0.05$) have been calculated comparing with the control (pH 8, Temp 23 °C, Sal 34 psu).

In the present work, the growth of *P. tricornutum* was significantly affected by all the pH combined treatments, and was influenced by temperature (Fig. 1). However, the CO_2 toxicity on microalgae is commonly a species-specific issue, with possible winners and losers (Hinga, 2002). Enhanced effects on different microalgae species growth under acidified conditions have been also reported, maybe caused by the stimulation of carbon fixation (Riebesell et al., 2007). High temperature may cause disruption of cell metabolism and death on microalgae (Richmond, 1986) but this environmental factor is quite species-specific, since higher heat tolerance species have also been described (Renaud et al., 2002). Salinity perturbations provoke different effects, from osmotic stress to change of the cellular ionic ratios, related to the selective ion permeability of the membrane (Kirst, 1989). But the cell density endpoint by itself is not strongly representative for testing toxicological effects on unicellular microalgae, since microalgal cells may be still viable even when they are not able to end their division cycle (González-Barreiro et al., 2006). Hence, cell viability analyses were also carried out. The principal symptom of cell death, whether from stress episodes or senescence, is related to the loss of membrane integrity (Cid et al., 1996) and thus to the inability to preserve homeostasis (Gahan, 1984). After 48 h of exposure, viability results showed slight lethal effects on some of the treatment combinations. Antioxidant defensive mechanisms may be responsible for these results as previously found for microalgae exposed to different toxicants (Chankova et al., 2014; Dewez et al., 2005; Esperanza et al., 2015).

FDA-based cell esterase activity test results provide an extra knowledge to that obtained with the PI assay, since FDA assay gives information on altered esterase activity (Seoane et al., 2014). In this study, metabolic activity did not show significant differences in most of the treatments with respect to the control. But this result could be species-specific and not necessarily related to the absence of acidification effects, as found in previous works (Bentley-Mowat, 1982; Franklin et al., 2001) where minimal uptake and hydrolysis of FDA in cultures of *P. tricornutum* was also reported. However, four of the treatments (pH 8, Temp 28 °C, Sal 40 psu; pH 7.4, Temp 28 °C, Sal 40; pH 6, Temp 28 °C, Sal 34 psu and pH 6, Temp 28 °C, Sal 40) showed a decrease of the metabolic activity. This decrease, which has been considered as a stress indicator (Franklin et al., 2001; Prado et al., 2012a), seems to be linked to the combination of low pH and high temperature and salinity. Water acidification may provoke cellular oxidative stress, causing redox misbalances and alterations on the protein structure, which is followed by a decline in the enzyme function and cellular activity (Prado et al., 2012b). Besides, the correlation between metabolic activity and photosynthetic activity has been defined in microalgae (Brookes et al., 2000). In this sense, the inhibition of metabolic activity could be linked to the damage at photosystem II (Esperanza et al., 2015) induced by the combination of pH, temperature and salinity.

As a result of the decrease in growth rate and metabolic activity of *P. tricornutum* cells, a decrease in cell volume was found on those cultures exposed to low pH (Temp 23 °C, Sal 34 psu). Cell apoptosis have been related to plasma membrane rupture and cell components spill (Darzynkiewicz et al., 1992), so non-viable cells should be recognized by their reduced size (Prado et al., 2011). In this case, effects on cell size seem to be mitigated under high salinity. An increase in cell volume has been also reported by other authors in different microalgae species as a response to different stressors (Cid et al., 1996; González-Barreiro et al., 2004; Seoane et al., 2017). Celular damage caused by high temperature and low pH may be responsible for the increase of signal detected on the side light scatter (SS) corresponding to intracellular complexity. The degradation of cell organelles is translated into an increase of cell complexity (Bray et al., 1993).

Alteration of cell autofluorescence has been established as a reliable endpoint in ecotoxicological studies with microalgae in order to assess the effects of several stress factors (González-Barreiro et al., 2004; Chalifour et al., 2009; Hadjoudja et al., 2009). Chlorophyll *a* fluorescence is a function of the cell pigment content and the photochemical activity of PSII in the photosynthetic electron transport chain (Franklin et al., 2001). The observed increase in chlorophyll *a* fluorescence suggested a decrease in the photosynthetic efficiency of cells, indicating an obstruction in the electron transport chain related to the inhibition of the PSII reaction centre in the acceptor side (Murthy et al., 1990; Cid et al., 1995). Also, changes on seawater chemistry as a result of acidification may enhance photosynthesis in marine microalgae (Johnson et al., 2013). Although previous studies with salinity stress have shown a significant inhibition of the photosynthetic activity (Endo et al., 1995; Liang et al., 2014), an increase of the PSII activity in *Dunaliella* (Liska et al., 2004) and *S. platensis* (Dhiab et al., 2007) has been also reported. In this case, acidification effects seem to be mitigated under high salinity.

The combined effects of CO_2 enrichment with temperature and salinity have been also reported using higher organisms. Oxidative stress has been found in clams submitted to low pH and high temperatures (Matozzo et al., 2013). Similarly, this interaction caused some physiological function problems on the mussel *Mytilus coruscus* (Wang et al., 2015). Evidences of synergy effects of exposure to low pH and salinity were found on the embryonic development of the amphipod *Echinogammarus marinus* (Egilsdottir et al., 2009). Interactive effects of pH, temperature and salinity were found in the larval growth rate of the Pacific oyster *Crassostrea gigas* (Ko et al., 2014) and in the mortality of the congeneric gastropods *Nassarius festivus* and *Nassarius conoidalis* (Zhang et al., 2014). However, some authors reported that elevated temperature mitigates the impact of seawater acidification (Brennand et al., 2010; Waldbusser et al., 2011). Undoubtedly, the interaction of acidification with other environmental stressors seems to cause species-specific responses (Byrne, 2011). In addition, it is important to take into account that results from single population research can lead to the over or underestimation of the impact of these environmental changes on species from different climatic regions (Calosi et al., 2017).

The findings of this work are of great importance since the drift of phytoplankton composition could be changed, with implications in the marine trophic networks (Moreno-Garrido et al., 1999; Forján et al., 2011; Sendra et al., 2017).

5. Conclusion

CO_2 enrichment in seawater is an important issue affecting marine ecosystems. However, there is a lack of information about the effects caused by this phenomenon when combined with other environmental stressors, such as temperature and salinity. Synergistic effects of the three parameters together were found on cell viability, cell size and autofluorescence, although these effects were not necessarily exacerbated by this combination, since high salinity seems to mitigate the effect of low pH. These results are of great importance in order to evaluate the potential risk of CO_2 enrichment and climate change consequences on marine phytoplankton.

Short and long term studies and the integration of results from species coming from different climatic regions and including marine and freshwater ecosystems would be useful to address the future consequences of climate change on phytoplankton communities and therefore, in aquatic environments.

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